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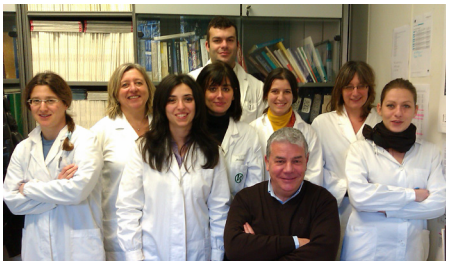
Sensitive *KRAS* Variant Detection by HRM on the ViiA™ 7 Real-Time PCR System



Clinical Biochemistry Unit, Department Of Clinical Physiopathology
University Of Florence, Florence, Italy

The Clinical Biochemistry Unit is mainly involved in the development and clinical validation of new potential biomarkers for the most common human solid cancers. The research group is particularly involved in the development of new strategies for the accurate and sensitive detection of target sequences in complex matrices.

Professor Claudio Orlando with the Clinical Biochemistry group (from the left): Francesca Salvianti, Pamela Pinzani, Serena Vinci, Lisa Simi, Nicola Pratesi, Francesca Malentacchi, Stefania Gelmini, and Irene Mancini.

Features	ViiA™ 7 Real-Time PCR System
Block configurations	96-well, Fast 96-well, 384-well (runs Fast or standard), TaqMan® Array Microfluidic Cards
Run time	30 minutes expected (Fast 96-well); 35 minutes (384-well)
Resolution	Down to 1.5-fold changes for singleplex reaction
Excitation source	OptiFlex™ System with halogen lamp
Detection channels	Decoupled—6 emission, 6 excitation
Security, auditing module	Optional software module
Remote monitoring	Available to monitor up to 4 instruments in real time and the status of up to 15 instruments
Data export format	User configurable: *.xls, * .xlsx, *.txt, and 7900 formats, as well as the new MIQE-compliant RDML format

Introduction

Somatic mutations of the *KRAS* gene can lead to a form of the protein that can direct cells to grow and divide without control, implicating KRAS in the development of several types of cancer. Moreover, *KRAS* mutants are capable of activating the epidermal growth factor receptor (EGFR) signaling pathway independently of EGFR activation, which may influence the action of drugs aimed at this pathway. Therefore, the development of fast and reliable tests to detect *KRAS* mutations is important to cancer researchers.

High resolution melt (HRM) analysis represents an efficient method to evaluate *KRAS* mutations that consist of a variety of nucleotide substitutions at different positions. Applications of HRM have been described for the identification of germ line and somatic mutations. Due to its high sensitivity, HRM is considered a valid approach to detect a minimal fraction of mutated cells in cancer tissues. This level of detection is, in many cases, not achievable by direct sequencing [1].

In this research-only study, an HRM mutation scanning experiment was performed on the Applied Biosystems® ViiA™ 7 Real-Time PCR System to screen DNA samples for single-base mutations at codons 12 and 13 of the *KRAS* gene. All 32 CRC samples had been previously run on the Applied Biosystems® 7900HT Real-Time System. Here we evaluate HRM reproducibility between the two real-time PCR systems.

Materials and methods

DNA isolation

DNA extraction from 32 CRC tissues snap-frozen in liquid nitrogen, as well as from cell lines, was performed using the QIAamp® DNA Mini Kit (Qiagen) following the manufacturer's protocol. DNA from CCRF-CEM, heterozygous for G12D mutation, and MCF-7, wild type DNA, were used as controls. DNA concentration was evaluated using NanoDrop® ND-1000 (Thermo Scientific).

Primer design

Primer sequences for HRM analysis of codons 12 and 13, selected taking into account the existence of a KRAS pseudogene, were: forward 5'-GTCACATTTTCATTATTTTTATTATAAGG-3' and reverse 5'-TTTACCTCTATTGTTGGATCATATTC-3'.

Real-time PCR and HRM analysis

For the amplification of the KRAS fragment (155 bp), which spans codons 12 and 13 of interest, 20 ng of DNA were amplified in a final volume of 20 µL using MeltDoctor™ HRM Master Mix (2X) (Applied Biosystems) and 300 nM of each primer.

Assays were performed in duplicate. To ensure reliable results, DNA from mutated and wild-type cell lines was included. Real-time PCR and HRM analyses were performed following the standard protocol provided by the manufacturer. The amplification was performed with an initial hold at 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Following amplification, a melt curve analysis was performed using protocols from the Applied Biosystems Guide to High Resolution Melting (HRM) with an annealing temperature of 60°C.

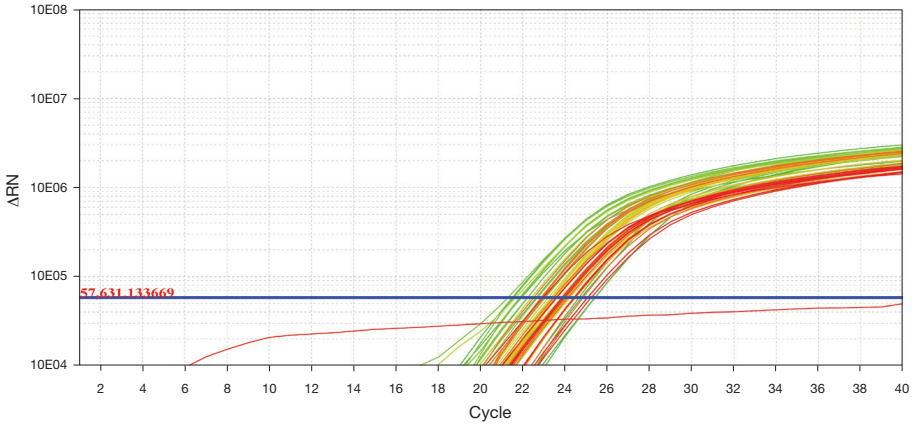


Figure 1. Amplification plot of KRAS assay.

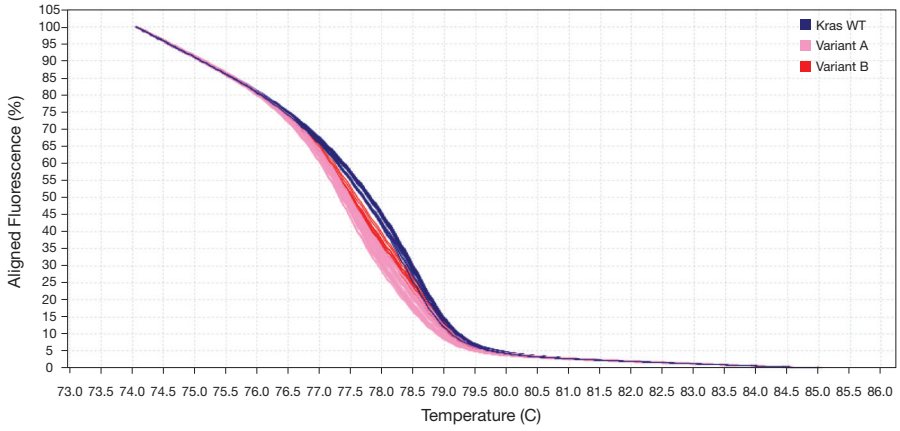


Figure 2. Aligned melt curve plot. The ViiA™ 7 Software clearly distinguished samples that did not show any mutated allele at codons 12 and 13 (*KRAS* WT) from mutated samples (Variant A and Variant B).

Results and discussion

Figures 1 through 3 show the HRM analysis performed on the ViiA™ 7 Real-Time PCR System. In the aligned melt curves, the ViiA™ 7 Software automatically identified three different variants: a wild-type cluster (KRAS WT), Variant A, and Variant B, both of which harbored a mutated allele at different percentages (Figure 2). The presence of a mutated allele was confirmed in all samples by direct sequencing.

Interestingly, the difference plot depicts different curve shapes for two samples (Figure 3). Although these two samples were grouped in the wild-type cluster, they show a different melting behavior from wild-type samples, compatible with the presence of a mutation in KRAS that was not confirmed by direct sequencing. Only after COLD-PCR amplification, an enrichment method for minority alleles [2], were we able to confirm, both with HRM and direct sequencing, the presence of a mutated allele in these two samples (results not shown).

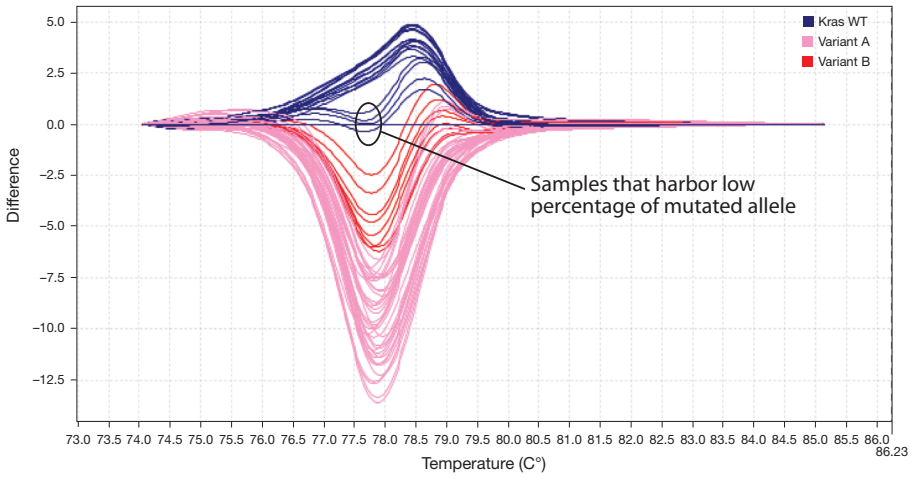


Figure 3. Difference plot showing sensitive KRAS variant detection. In the group of WT KRAS there are two samples that show a different melting profile. These two samples contain a low percentage of mutated allele.

Conclusions

Our study confirms that HRM analysis performed on the ViiA™ 7 Real-Time PCR System provides an accurate and sensitive method that enables the detection of very low percentages of mutated alleles in tumor samples. Prescreening by HRM analysis is a useful method to reveal low-represented *KRAS* mutations harbored by a small cellular subset of samples.

Previous results obtained with the 7900HT Real-Time PCR System are comparable to the results obtained in this study. Moreover, the new ViiA™ 7 Real-Time PCR software interface was innovative and easy to use, characterized by marked ability to detect samples with low-level mutations.

MeltDoctor™ HRM Reagents

Applied Biosystems® MeltDoctor™ HRM Reagents were developed and optimized solely for high resolution melt analysis. MeltDoctor™ Reagents employ MeltDoctor™ HRM Dye, a stabilized form of SYTO®-9 dye—a next-generation Molecular Probes® dsDNA-binding dye. The MeltDoctor™ HRM Dye possesses significant optical and chemical properties important for high-performance HRM including:

- Low background fluorescence
- High brightness in the presence of double-stranded DNA
- Minimal temperature shift of DNA melting due to dye binding
- Thermal stability to tolerate PCR cycling conditions
- No inhibition of polymerase activity, resulting in high PCR efficiency

MeltDoctor™ HRM Master Mix contains all components needed for HRM (excluding template and primers) and is formulated for superior HRM performance across a wide range of genomic targets. When more flexibility in reactions is needed, the MeltDoctor™ HRM Reagent Kit provides enzyme, fluorescent dye, and dNTP mix separately. Both reagent sets utilize AmpliTaq Gold® 360 DNA Polymerase, a hot start–enabled DNA polymerase that minimizes nonspecific product formation and enables room temperature reaction setup. In addition, the dNTP blend includes dUTP, which minimizes carryover contamination by allowing amplicon degradation by uracil DNA glycosylase (UDG) in subsequent PCR reactions.

To learn more about MeltDoctor™ HRM Reagents, go to www.appliedbiosystems.com/hrm.

To learn more about performing HRM experiments, or to download the Applied Biosystems *HRM Getting Started Guide and A Guide to High Resolution Melting (HRM) Analysis*, go to www.appliedbiosystems.com/hrm.

References

1. Simi L, Pratesi N, Vignoli M, et al. (2008) High-resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer. *Am J Clin Pathol* 130:247–253.

2. Mancini I, Santucci C, Sestini R et al. (2010) The use of COLD-PCR and high-resolution melting analysis improves the limit of detection of KRAS and BRAF mutations in colorectal cancer. *J Mol Diagn* 12(5):705-711.

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